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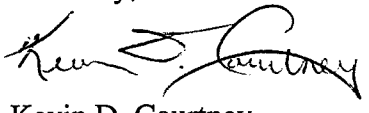

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Abl-interactor (Abi) proteins bind and are phosphorylated by the non-receptor tyrosine kinases Abl and Arg. Two Abi family genes, *Abi-1* and *Abi-2*, have been identified. Abi proteins demonstrate properties consistent with a potential tumor suppressor function. We investigated the role of Abi-1 and Abi-2 proteins and their interactions with c-Abl and c-Abl-derived oncogenes in normal development and tumorigenesis. Abi-1 and Abi-2 exhibit both unique and overlapping temporal-spatial patterns of expression during embryonic and post-natal mouse development. Abi-2 is enriched in regions of the central and peripheral nervous systems (CNS and PNS) pre- and post-natally. Abi-1 is also enriched in regions of the post-natal brain, but is not enriched in the pre-natal CNS and is absent from examined PNS structures. Abi proteins undergo changes in phosphorylation during development. Overexpression of fluorescent-tagged Abi-1 and Abi-2 proteins in fibroblasts and primary neurons reveals a cytosolic, punctate localization. Examination of Abi proteins in the presence of oncogenic forms of Abl and Src revealed loss of Abi expression due to ubiquitin-mediated degradation. Abi degradation was not observed in other tumor settings, including breast cancer-derived cell lines overexpressing members of the erbB family of receptor tyrosine kinases.

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Introduction

Abl-interactor (Abi) proteins are substrates and adaptors for the non-receptor tyrosine kinases (NRTKs) Abl and Arg. Two Abi family genes, *Abi-1* and *Abi-2*, have been identified, both with multiple splice variants (1-5). Abi proteins interact with Abl and Arg through a carboxy-terminal SH3 domain and through proline rich sequences. Abi proteins also contain a homeodomain homology region (HHR) with sequence similarity to the DNA binding domain of homeobox-containing proteins (Appendix, Fig. 1). Mutations in Abi-2 are capable of activating c-Abl transforming capacity, and overexpression of truncated Abi-1 in NIH3T3 cells suppresses v-Abl transforming ability (1, 2). We hypothesize that Abi-proteins may function as potential tumor suppressors. The purpose of this study is to investigate the role of Abi-family proteins and their interactions with the c-Abl tyrosine kinase and *c-Abl*-derived oncogenes in normal development and transformation. To provide a framework for the normal functions of Abi-1 and Abi-2 proteins, we have examined Abi expression, phosphorylation, and subcellular localization during embryogenesis and post-natal development. We have investigated the fate of Abi proteins in the presence of Abl- and Src NRTK- derived oncogenes. Oncogenic forms of Abl and Src elicit the ubiquitin-mediated degradation of Abi proteins. We have compared Abi expression in paired human normal and tumorigenic tissue samples from breast, lung, liver, and colon, as well as in breast cancer- derived cell lines which overexpress various erbB RTK family members. Ongoing and future studies are directed at further defining the subcellular localization of Abi proteins and identifying novel Abi interacting proteins.

Annual Summary

This summary describes the research accomplishments achieved as relate to the Tasks outlined in the Statement of Work for Grant Number DAMD17-98-1-8069.

Task: Determine the pattern of expression and tyrosine phosphorylation status of Abi-1, Abi-2, and c-Abl throughout mouse embryogenesis and post-natal development.

We examined Abi-1, Abi-2, and c-Abl protein expression throughout mouse development. Lysates of mouse embryos of gestational age 10-16 days (E10-E16) were prepared and analyzed by immunoprecipitation and Western blotting techniques. Peak c-Abl expression was observed at the earlier ages examined (E10-E13), consistent with previous reports of *c-Abl* transcript levels (6). Multiple bands were recognized for both Abi-1 and Abi-2 proteins, possibly reflecting multiple splice variants and/or post-translational modifications such as phosphorylation and ubiqutination. Abi and c-Abl protein levels were higher in lysates prepared from embryo heads than trunks. Among post-natal tissues examined, highest Abi expression was observed in the brain. In post-natal brain lysates, Abi proteins underwent a shift in apparent molecular weight to faster migrating forms on reducing gels with increasing age of the mice. Potato acid phosphatase treatment revealed Abi phosphorylation in neonatal brains. c-Abl expression was also higher in brains of neonates than in older mice.

To more precisely localize Abi-1 and Abi-2 expression during development, we performed *in situ* hybridization to detect *Abi-1* and *Abi-2* transcripts at embryonic days 10, 12, and 16. *Abi-2* message was most prominent in the developing central nervous system (CNS) and dorsal root ganglia (DRGs) of the peripheral nervous system (PNS). Elevated *Abi-2* mRNA levels were observed in regions of both pre- and post-mitotic neural cells in the developing brain. *Abi-1* transcript levels appeared fairly uniform throughout the embryo. *Abi-1* message was present in the developing CNS, but not elevated relative to levels in other tissues. Unlike *Abi-2*, *Abi-1* mRNA was not detected in DRGs.

These results suggested Abi-1 and Abi-2 might exhibit both unique and overlapping functions in the developing nervous system. We subsequently examined their expression patterns in the developing post-natal mouse brain from birth through maturity. *Abi-1* and *Abi-2* message showed very similar patterns of expression at all post-natal ages studied. Sites of prominent *Abi-1* and *Abi-2* expression included the hippocampus, neocortex, Purkinje layer of the cerebellum, and the mitral cell layer of the olfactory bulb. Abi-1 and Abi-2 therefore appeared enriched in projection neuron populations and regions which exhibit synaptic plasticity.

We next examined the subcellular localization of Abi proteins by employing immunocytochemistry and fractionation techniques. In collaboration with Antonius and Margon Vandongen in the Department of Pharmacology and Cancer Biology, we transfected cultured primary hippocampal neurons or fibroblasts with fusion constructs of Abi-1 and Abi-2 with enhanced yellow fluorescent protein (EYFP). Abi-1•EYFP and Abi-2•EYFP were expressed in a punctate pattern and excluded from the nucleus. In the transfected neurons this cytosolic, vesicular pattern of expression was observed in the cell body and in neurites. Abi and c-Abl protein expression was observed by Western blot analysis in growth cone particles prepared from fractionated neonatal rat brains. Fractionation of adult rat brains revealed the presence of Abi and c-Abl in synaptosomes.

Future efforts to further characterize the subcellular localization of Abi-1 and Abi-2 will involve colocalization studies employing confocal microscopy and protein markers for specific subcellular compartments. Given the punctate, extra-nuclear pattern of expression for Abi-1 and Abi-2, these proteins may reside in association with the endoplasmic reticulum (ER) or Golgi network. We will therefore attempt to colocalize Abi-1 and Abi-2 with known ER and Golgi markers. We will also treat cultured cells with agents which

disrupt these organelles and follow the fates of Abi-1•EYFP and Abi-2•EYFP by fluorescent microscopy to determine whether Abi protein distribution is altered after treatment. Treatment with the fungal metabolite brefeldin A, which alters the morphology of the Golgi and trans-Golgi network, has been shown to lead to the redistribution of a number of Golgi proteins (7). The pattern of ER-associated protein localization may be altered by colchicine treatment, which depolymerizes microtubules and therefore disrupts the microtubule cytoskeleton along which ER tubules align (8, 9). Subcellular fractionation experiments to isolate ER and Golgi components will be carried out in conjunction with immunofluorescence studies of cultured cells.

A parallel aim is to identify proteins that interact with Abi-2. We will screen a human brain cDNA library for proteins that bind Abi-2 using the yeast two-hybrid technique (10). The yeast two-hybrid system makes use of both nutritional and colorimetric reporters. Yeast which require supplemental histidine, leucine, and tryptophan to survive are co-transformed with a bait protein, in this case a fusion of the Gal4 DNA binding domain and the homeodomain homologous region (HHR) of Abi-2, and a target, here a fusion of the Gal4 activation domain and library cDNAs. Interaction between the bait and the target will lead to transcriptional activation of a *HIS3* reporter, allowing yeast to grow in the absence of supplemental histidine, and a *lacZ* reporter, conferring a blue color to yeast in a β-galactosidase assay (10). Positive interactions will be independently confirmed and the sites of interaction determined via *in vitro* binding assays.

Task: Ascertain whether Abi protein expression or phosphorylation status is altered in cancer.

(A portion of the work which was carried out to address this aim is presented in the appended article Dai, Z., Quackenbush, R.C., **Courtney, K.D.**, Grove, M., Cortez, D., Reuther, G.W., and Pendergast, A.M. (1998) "Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway" *Genes & Development* 12:1415-1424.)

We investigated the fate of Abi proteins in the setting of various cancers. As Abi proteins are known to interact with the c-Abl tyrosine kinase, we first examined Abi proteins in the presence of the *Bcr-Abl* oncogenes. Bcr-Abl proteins P185, P210, and P230 result from a 9;22 chromosomal translocation which generates the Philadelphia chromosome (Ph^1) (11). Bcr-Abl proteins are constitutively activated tyrosine kinases and are associated with leukemogenesis. We showed that Bcr-Abl downregulates Abi protein expression. Infection of mouse or human myeloid cells or mouse bone marrow with Bcr-Abl-expressing retrovirus elicited the loss Abi proteins following the onset of detectable Bcr-Abl expression. Examination of Ph^1-positive leukemic cell lines and bone marrow from patients with Ph^1-positive leukemias also revealed loss of Abi proteins. We determined by RNase protection assay and reverse transcriptase polymerase chain reaction (RT-PCR) that *Abi-1* and *Abi-2* transcripts were present and their levels unaltered in cells expressing Bcr-Abl. This suggested a post-translational mechanism for Bcr-Abl-mediated Abi degradation. The ubiquitin-dependent proteasome pathway had been shown to regulate the levels of other proteins by conjugating ubiquitin chains at lysine residues, thereby flagging the target protein for degradation (12). Bcr-Abl was found to mediate Abi protein downregulation in a ubiquitin-dependent manner. Abi proteins were ubiquitinated, and proteasome inhibition lead to the accumulation of Abi proteins of increased molecular weight, consistent with Abi ubiquitination.

We next wanted to determine whether Abi degradation was elicited by oncogenes other than Bcr-Abl. Abi proteins were not degraded in Ph^1-negative leukemic cell lines and bone marrow from a patient with Ph^1-negative acute myelogenous leukemia. Abi protein levels also appeared unaltered by Western blot analysis of human lung, liver, colon, and breast carcinoma tissues as compared to paired normal tissues. However, expression of v-

Src, a constitutively active, oncogenic non-receptor tyrosine kinase, in BaF3 mouse myeloid cells promoted the ubiquitin-dependent degradation of Abi proteins.

We subsequently examined Abi protein levels in breast cancer cell lines overexpressing type 1 growth factor receptor tyrosine kinases, or erbB proteins, acquired from Lyndsay Harris in the Department of Medicine at Duke University Medical Center. Four erbB family members have been identified: erbB1 (epidermal growth factor receptor (EGFR)), erbB2 (HER2/Neu), erbB3, and erbB4 (13, 14). Ligand binding induces homo- and hetero-dimerization of erbB receptors in nine possible combinations. Six EGF-like ligands have been identified for erbB1, while erbB3 and erbB4 bind neuregulins (13). No ligand has been identified for erbB2. Overexpression of erbB2 has been correlated with poorer prognosis for patients with breast carcinoma (13, 14). EGF-dependent activation of Src-family kinases has been demonstrated in cells overexepressing erbB1 (15, 16). We hypothesized that overexpression of erbB family receptors in breast cancer cell lines might lead to Abi protein degradation in a Src-dependent manner. We tested various cell lines overexpressing each of the erbB receptors for Abi degradation. Abi loss was not detected in these cell lines. Future work will examine Abi protein levels following ligand stimulation of these cells. In addition we will also examine Abi protein levels in breast cancer cell lines and primary tumors that co-overexpress both erbB family members and c-Src. These cells and tissues will be requested from Sarah Parsons at the University of Virginia.

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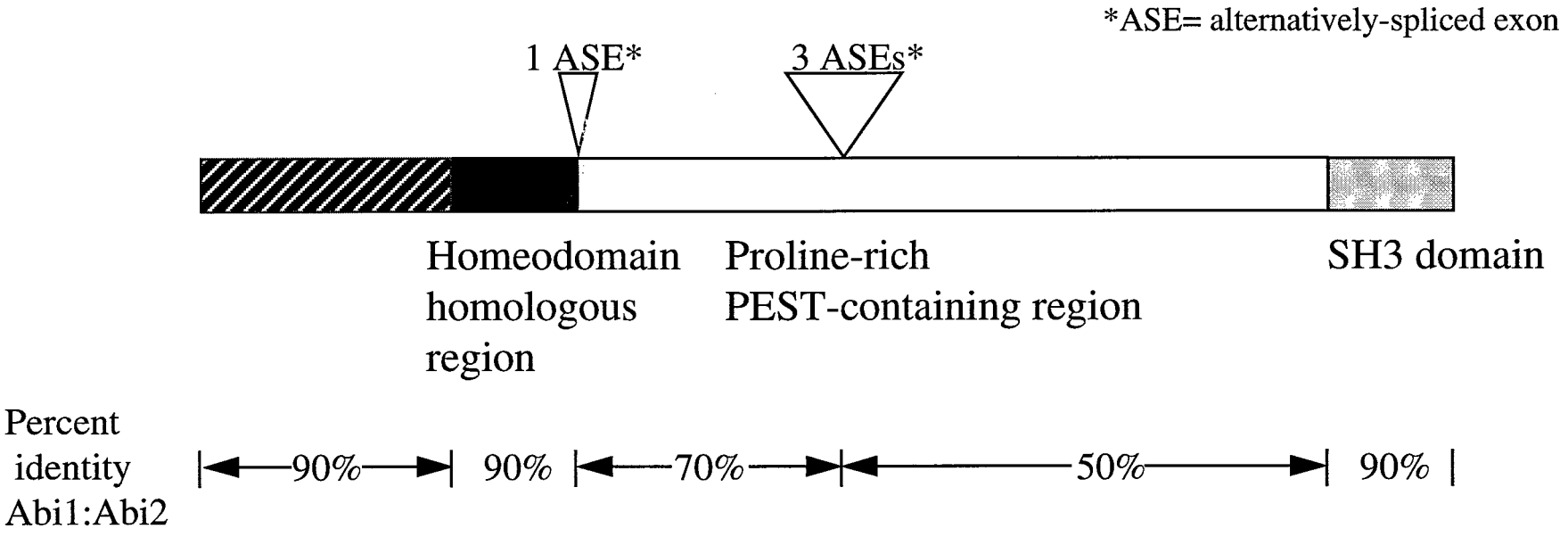
Figure 1: Structure and Homology of Abi1 and Abi2 Proteins



Key Research Accomplishments: as described for the Tasks provided in the Body of this application

I. Determine the pattern of expression and tyrosine phosphorylation status of Abi-1, Abi-2, and c-Abl throughout mouse embryogenesis and post-natal development.

- Localized *Abi-1* and *Abi-2* transcripts by in situ hybridization throughout mouse development
- Determined Abi and Abl protein levels and phosphorylation status at different stages of mouse development employing techniques of immunoprecipitation, Western blotting, and phosphatase treatment
- Subcellular localization of endogenous Abi, Abl, and Arg proteins in rat brain fractions by Western blot analysis
- Subcellular localization of enhanced yellow fluorescent protein (EYFP) tagged Abi-1 and Abi-2 in cultured fibroblasts and primary neurons by transfection, immunocytochemistry, and confocal microscopy techniques

II. Task: Ascertain whether Abi protein expression or phosphorylation status is altered in cancer.

- Determined Abi proteins are degraded in a ubiquitin-dependent manner by Bcr-Abl and v-Src
- Confirmed by RNase protection and RT-PCR assays that *Abi-1* and *Abi-2* transcripts are generated in the presence of Bcr-Abl
- Abi protein degradation was observed in Ph^1+ leukemia-derived cells, but not in Ph^1- leukemias or in human breast, colon, lung, or liver tumors
- Abi proteins were not degraded in untreated breast cancer derived cell lines overexpressing various erbB receptors

Reportable Outcomes:

I. Manuscripts:

Dai, Z., Quackenbush, R.C., **Courtney, K.D.**, Grove, M., Cortez, D., Reuther, G.W., and Pendergast, A.M. (1998) "Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway" *Genes & Development* 12:1415-1424.

II. Abstracts/Presentations:

Courtney, K.D., Zipfel, P.A., Grove, M., Freameau, R.T., LaMantia, A.-S., and Pendergast, A.M. "Expression of the Abl-interactor (Abi) genes and the Abl proto-oncogene during mouse embryogenesis and post-natal development." Presented at the Fourteenth Annual Meeting on Oncogenes, June 24-27, 1998.

Abstract for poster presentation at the Fourteenth Annual Meeting on Oncogenes, June 24-27, 1998.

EXPRESSION OF THE ABL-INTERACTOR (ABI) GENES AND THE ABL PROTO-ONCOGENE DURING MOUSE EMBRYOGENESIS AND POST-NATAL DEVELOPMENT

Kevin D. Courtney[1], Patricia A. Zipfel[1], Matthew Grove[1], Robert T. Fremeau[1,2], Anthony-Samuel LaMantia[2], and Ann Marie Pendergast[1]. [1]Department of Pharmacology and Cancer Biology and [2]Department of Neurobiology, Duke University Medical Center, Durham, NC 27710

We have previously identified and cloned an Abl-interactor protein, Abi-2, which binds to and is phosphorylated by the c-Abl tyrosine kinase. Both Abi-2 and the related Abi-1 protein bind to c-Abl and to the Abl-related (Arg) tyrosine kinase. Abi-1 and Abi-2 are the products of different genes. Significantly, Abi-1 and Abi-2 have been shown to regulate the transforming capacity of Abl proteins. The roles of c-Abl and Abi proteins in normal cell growth and differentiation remain poorly understood. The objective of this study was to examine the expression of c-Abl and Abi proteins during mouse embryogenesis and post-natal development. Immunoprecipitation and Western blot analysis identified c-Abl and Abi proteins in mouse embryo lysates as early as embryonic day 10 (E10). Levels of c-Abl decreased as embryos approached full gestation. Abi proteins show increasing amounts of higher molecular weight forms with progression from E10-E13. Although Abi proteins were ubiquitously expressed, they were highly expressed in the brain. Abi protein expression was greater in lysates derived from embryonic heads than from trunks. *In situ* hybridization revealed *abi-1* transcripts to be prominent throughout the central nervous system (CNS) during embryonic and post-natal development. *Abi-2* message also appeared enriched in the CNS and peripheral nervous system throughout development, with apparent concentrations in specific neuronal populations, including dorsal root ganglia at E16, the Purkinje cell layer of the cerebellum, and Ammon's horn and the dentate gyrus of the hippocampus. A downward shift in apparent molecular weight with increasing age was observed for Abi proteins detected in brain lysates prepared from late embryonic and post-natal mice. This shift to faster migrating forms with age corresponded in part with loss of phosphorylation of Abi proteins in brain lysates from older mice. In cultured mouse embryonal carcinoma P19 cells, Abi protein mobility was altered following treatment with retinoic acid, which induces neuronal differentiation. These data suggest that Abi expression may be developmentally regulated and that Abi proteins may play a role in c-Abl mediated signal transduction in the brain.

Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway

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Oncogenic forms of the Abl and Src tyrosine kinases trigger the destruction of the Abi proteins, a family of Abl-interacting proteins that antagonize the oncogenic potential of Abl after overexpression in fibroblasts. The destruction of the Abi proteins requires tyrosine kinase activity and is dependent on the ubiquitin–proteasome pathway. We show that degradation of the Abi proteins occurs through a Ras-independent pathway. Significantly, expression of the Abi proteins is lost in cell lines and bone marrow cells isolated from patients with aggressive Bcr–Abl-positive leukemias. These findings suggest that loss of Abi proteins may be a component in the progression of Bcr–Abl-positive leukemias and identify a novel pathway linking activated nonreceptor protein tyrosine kinases to the destruction of specific target proteins through the ubiquitin–proteasome pathway.

[*Key Words:* Abi; Bcr–Abl; v-Src; ubiquitin-dependent proteolysis; Ph[1]-positive leukemia]

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Ubiquitin-dependent proteolysis is a critical component of diverse biological processes including cell cycle progression, the immune response, embryonic development, protein transport, and apoptosis (for review, see Deshaies 1995; Hochstrasser 1995; King et al. 1996; Varshavsky 1997). Here we show that ubiquitin-dependent proteolysis may also play a role in oncogenesis by activated nonreceptor tyrosine kinases.

Oncogenic forms of the Abl tyrosine kinase are linked to the development of human, murine, and feline leukemias (Bergold et al. 1987; Rosenberg and Witte 1988; Laneuville 1995; Gotoh and Broxmeyer 1997). Activation of cellular Abl (cAbl) oncogenic potential may occur as a consequence of chromosomal translocation events that generate chimeric fusion proteins such as Bcr–Abl and Tel–Abl (Gotoh and Broxmeyer 1997). The Bcr–Abl tyrosine kinases are produced by a reciprocal t(9;22)(q34;q11) chromosomal translocation that gives rise to the Philadelphia chromosome (Ph[1]). The translocation fuses varying amounts of the *Bcr* gene on chromosome 22 with sequences upstream of the second exon of the *c-Abl* gene on chromosome 9. Three different Bcr–Abl fusion proteins may be produced. The 210-kD form of Bcr–Abl (p210) is the causative agent of >95% of human chronic myelogenous leukemia (CML) cases (Clarkson et al. 1997; Gotoh and Broxmeyer 1997). The 185-kD Bcr–Abl protein (p185) is associated with a subset of acute lymphocytic leukemia (ALL) (Kurzrock et al. 1987). Recently, a rare 230-kD Bcr–Abl protein (p230) has been detected in patients with chronic neutrophilic leukemia (CNL) (Wada et al. 1995; Melo 1996). The p185 and p210 Bcr–Abl proteins have been shown to elicit ALL- and CML-like syndromes in mice, respectively, and to transform fibroblasts and hematopoietic cells in culture (Daley et al. 1990; Heisterkamp et al. 1990). Several mechanisms have been proposed to explain how Bcr–Abl transforms cells. Among these are increased resistance to apoptosis, enhanced proliferative capacity, defective adhesion, and increased motility of the Bcr–Abl-expressing cells (Clarkson et al. 1997; Cortez et al. 1997; Gotoh and Broxmeyer 1997; Salgia et al. 1997). The biological effects of Bcr–Abl require the constitutive tyrosine kinase activity of the chimeric protein. Multiple proteins have been identified as downstream targets of Bcr–Abl. Among these are proteins involved in the regulation of mitogenic and apoptotic pathways as well as cytoskeletal-associated proteins. Primarily, components of the Ras and phosphatidylinositol 3-kinase (PI3k) pathways are critical for Bcr–Abl-dependent transformation. Dominant interfering mutants of Grb-2, Ras, and c-Jun block Bcr–Abl-mediated transformation (Gishizky et al.

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1995; Raitano et al. 1995; Sawyers et al. 1995; Cortez et al. 1996). Grb-2, Ras, and c-Jun are components of the same signaling pathway, thereby emphasizing the essential role of this pathway in the transmission of the Bcr–Abl-transforming signal. Also, inhibition of PI3k and its downstream target, the Akt serine kinase, decreases transformation by Bcr–Abl (Skorski et al. 1995, 1997). In addition, interfering with the function of the transcription factors, c-Myc and NF-κB, abolishes transformation by Bcr–Abl (Sawyers et al. 1992; Reuther et al. 1998).

Less clear is the contribution of other downstream protein targets to Bcr–Abl-dependent transformation. Although the levels of the anti-apoptotic Bcl-2 mRNA and protein are elevated in some Bcr–Abl-expressing cells (Sanchez-Garcia and Grutz 1995), it is unclear whether Bcl-2 is up-regulated in all Bcr–Abl-expressing cells, and whether its up-regulation is necessary and sufficient for the anti-apoptotic activity of Bcr-Abl (Cortez et al. 1996). Likewise, it is not known what role the increased tyrosine phosphorylation of a number of cytoskeletal-associated proteins has on the altered adhesion and the overall transforming properties of Bcr–Abl-positive cells (Salgia et al. 1995).

Although numerous targets for the Abl kinases have been identified, only a few of these have been shown to be important in modulation of the Abl-transforming potential (Gotoh and Broxmeyer 1997). Recently, we and other researchers identified a family of Abl-interactor (Abi) proteins that bind specifically to both the SH3 and carboxy-terminal proline-rich sequences of Abl (Dai and Pendergast 1995; Shi et al. 1995). Two distinct, yet highly related genes, *abi-1* and *abi-2*, were identified and cloned. The corresponding protein products share overall 69% identity with the greatest homology observed in the amino-terminal homeobox-like domain, proline-rich sequences, and the carboxy-terminal SH3 domain. The Abi proteins are substrates of the Abl kinases. Significantly, Abi proteins antagonize the oncogenic activity of Abl in fibroblasts. Overexpression of Abi-1 potently suppresses the transforming activity of viral Abl (v-Abl) in NIH-3T3 fibroblasts (Shi et al. 1995). Furthermore, coexpression of a truncated form of Abi-2 with c-Abl activates the oncogenic potential of c-Abl (Dai and Pendergast 1995). These and other data (Wang et al. 1996; Biesova et al. 1997) suggest that the full-length Abi proteins may function as growth inhibitors in mammalian cells.

Inactivation of molecules that function as growth inhibitors/tumor suppressors is a common event in a large number of cancers (Cordon-Cardo 1995). As illustrated for p53 and pRb, the activity of the tumor suppressors may be abrogated by mutations of the corresponding DNAs or by the sequestration of the tumor suppressor proteins by specific viral or cellular proteins. Increasing evidence is accumulating that implicates selective proteolysis in the functional inactivation of tumor suppressor proteins (Deshaies 1995; Haupt et al. 1997; Kubbutat et al. 1997). In particular, ubiquitin-dependent proteolysis appears to play a role in this process. Protein degradation by the ubiquitin pathway involves the covalent attachment of multiple ubiquitin polypeptides to the substrate protein, followed by the degradation of the polyubiquitinated substrate by the 26S proteasome, a large ATP-dependent multienzyme complex (Varshavsky 1997). Several proteins that function as growth inhibitors/tumor suppressors have been reported to be degraded through ubiquitin-dependent proteolysis. Among these are p53 and the cyclin-dependent kinase inhibitors Sic1p, Far1p, and p27 (Pagano et al. 1995; Feldman et al. 1997; Haupt et al. 1997; Henchoz et al. 1997; Kubbutat et al. 1997; Skowyra et al. 1997; Verma et al. 1997).

Here we show that oncogenic forms of the Abl and Src nonreceptor tyrosine kinases elicit the destruction of the Abi proteins by the ubiquitin-dependent proteasome machinery. The elimination of the Abi proteins by the oncogenic tyrosine kinases occurs through a novel Ras-independent pathway that is initiated by the constitutive tyrosine kinase activity of the oncoproteins. Significantly, the expression of Abi proteins is lost in cell lines and bone marrow cells from Ph^1-positive leukemia patients. These findings suggest that loss of Abi proteins may play a role in oncogenesis and implicates ubiquitin-dependent proteolysis in tumor progression.

Results

Bcr–Abl down-regulates Abi expression in hematopoietic cells

To understand the role of Abi proteins in Abl-mediated transformation, we investigated the expression of Abi proteins in normal hematopoietic cells as well as in cells transformed by the oncogenic Bcr–Abl fusion protein. The *Bcr–Abl* gene was introduced into the pro-B BaF3 cell line and the multipotent myeloid progenitor 32D cell line. Both cell types are dependent on interleukin 3 (IL-3) for growth and survival. Expression of Bcr–Abl in BaF3 and 32D cells induces cellular transformation, confers cytokine-independent growth, and blocks apoptosis (Cortez et al. 1995, 1996, 1997). Abi proteins are expressed in BaF3 and 32D cells and migrate as a doublet of 60 and 65 kD (Fig. 1A, lane 1). Because of the strong homology between Abi 1 and Abi 2, the available anti-Abi antibodies cannot distinguish between the two proteins and therefore, we will refer to these protein bands as Abi. Surprisingly, little if any Abi protein could be detected in BaF3 cells transformed with p185 Bcr–Abl (Fig. 1A, lanes 2,3). Similar findings were obtained in 32D cells transformed by Bcr–Abl. These results indicate that the expression of the oncogenic Bcr–Abl tyrosine kinase down-regulates the expression of Abi proteins. Consistent with this notion, the expression of HA-tagged exogenous Abi 2 in 32D cells is also down-regulated by Bcr–Abl in the same manner as that of the endogenous Abi proteins (data not shown). The Bcr–Abl-mediated down-regulation of Abi expression requires Bcr–Abl tyrosine kinase activity, as the expression of the kinase-deficient mutant p185 K671R Bcr–Abl (Cortez et al. 1995) failed to down-regulate Abi expression (Fig. 1A, lane 4). To determine whether the loss of Abi expression in Bcr–Abl-transformed hematopoietic cells is caused by

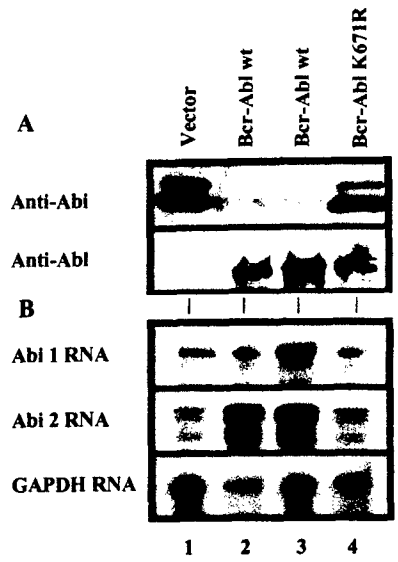

Figure 1. The oncogenic Bcr–Abl tyrosine kinase down-regulates Abi protein expression in BaF3 cells. (*A*) Loss of Abi protein with p185Bcr–Abl expression. BaF3 cells (2×10^6) were infected (lanes *1,3,4*) with the indicated retroviral expression vectors or stably transfected (lane *2*) with the indicated plasmid. Abi proteins were detected by immunoblotting with polyclonal 5421 anti-Abi antibodies (*top*). Bcr–Abl (lanes *2,3*) or Bcr–Abl (K671R) (lane *4*) protein was detected by immunoblotting with monoclonal anti-Abl antibody (*lower*). (*B*) Bcr–Abl expression does not alter *abi 1* or *abi 2* mRNA levels. *abi 1*, *abi 2*, or *GAPDH* antisense RNA probes were protected against RNase digestion by BaF3 total RNA. ^{35}S-Labeled protected probes were electrophoresed on acrylamide/urea gels and detected by autoradiography.

a decrease in the transcription of *abi* genes, Northern blot analysis and RNase protection assays were performed. BaF3 cells express message for both *abi 1* and *abi 2* (Fig. 1B, lane 1). No significant change in the *abi 1* and *abi 2* mRA levels, however, was observed among cells expressing the vector control, wild-type p185 Bcr–Abl, or the p185 K671R Bcr–Abl mutant (Fig. 1B, cf. lanes 1–4). This suggests that Bcr–Abl down-regulates *abi* expression by a mechanism other than transcriptional regulation.

Bcr–Abl down-regulates Abi expression through the ubiquitin–proteasome pathway

The oncogenic Bcr–Abl proteins down-regulate Abi expression without significantly reducing the level of *abi* transcripts. This suggests that the down-regulation may occur post-translationally. An increasing number of cellular processes have been shown to be critically dependent on the control of protein abundance catalyzed by the ubiquitin-dependent proteasome pathway (Deshaies 1995). Therefore, we tested whether the down-regulation of Abi expression by Bcr–Abl uses this pathway. First, we examined whether the down-regulation of Abi proteins by Bcr–Abl is caused by increased instability of the proteins. To this end we synthesized ^{35}S-labeled Abi 2 in vitro using a rabbit reticulocyte lysate (RRL) and tested its stability in the presence or absence of in vitro-translated Bcr–Abl. The RRL system has been used commonly as the source of active ubiquitinating enzymes and proteasome complexes (Nielsen et al. 1997; Pagano et al. 1997). In the absence of Bcr–Abl, Abi 2 was relatively stable. In contrast, in the presence of Bcr–Abl Abi 2 was degraded rapidly in the RRL (Fig. 2, cf. lanes 3 and 6). This suggests that Bcr–Abl increases Abi protein instability. Consistent with this, we found that addition of ATPγS, a nonhydrolyzable ATP analog that prevents degradation of ubiquitinated proteins by the proteasome but does not prevent their ubiquitination, blocked Abi 2 degradation in RRL system (Fig. 2, lane 7).

We then tested whether the down-regulation of Abi proteins by Bcr–Abl in hematopoietic cells is dependent on the ubiquitin–proteasome pathway. BaF3 cells transfected with either a control vector or an expression vector for p185 Bcr–Abl were treated with two specific inhibitors of the ubiquitin–proteasome machinery, LLnL (*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal) and lactacystin (Aberle et al. 1997). As shown by Western blot analysis, LLnL and lactacystin inhibited the down-regulation of Abi expression in Bcr–Abl-expressing cells (Fig. 3A, lanes 5,6). Treatment of BaF3 cells expressing p185 Bcr–Abl with LLnL and lactacystin resulted in the accumulation of 60- and 65-kD Abi proteins, as well as immunoreactive Abi proteins with a slower mobility in SDS gels. The slower mobility bands may represent Abi proteins that are modified during the process of ubiquitin-mediated proteolysis. Interestingly, the Abi proteins, in particular the slower mobility forms, also accumulated to higher levels in the control BaF3 cells treated with LLnL and lactacystin (Fig. 3A, lanes 2,3). This suggests that Abi expression in normal cells may also be regulated, at least in part, by ubiquitin-mediated proteolysis. We then examined whether Abi proteins are targets for ubiquitination. A plasmid expressing an HA-tagged Abi 2 was transfected into Bosc 23 cells in the

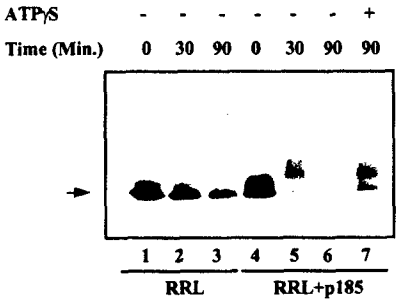

Figure 2. In vitro degradation of Abi 2 is stimulated by p185Bcr–Abl and is ATP dependent. Abi 2 was synthesized in the presence of [^{35}S]methionine using a coupled transcription–translation kit (Promega). The labeled Abi 2 was incubated in a protein-degradation reaction mix with or without unlabeled p185Bcr–Abl, and with or without ATPγS, as indicated. Samples (equal volume) were removed at indicated time points and analyzed by SDS-PAGE and fluorography.

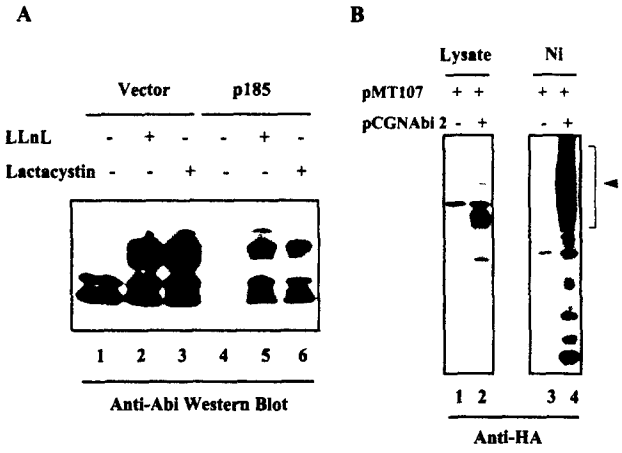

Figure 3. Bcr–Abl down-regulates Abi expression through an ubiquitin-dependent proteolysis pathway. (*A*) Proteasome-specific inhibitors LLnL and lactacystin inhibit Bcr–Abl-induced Abi down-regulation. BaF3 cells transfected by vector alone (lanes *1–3*) or expression vector encoding p185 Bcr–Abl (lanes *4–6*) were untreated (lanes *1,4*) or treated with 50 μM LLnL (lane *2,5*) or 10 μM lactacystin (lanes *3,6*) for 8 hr. Cells (2×10^6) were lysed in SDS sample buffer and subjected to Western blot analysis with 5421 anti-Abi antibodies. (*B*) Abi 2 is ubiquitinated in Bosc 23 cells. Bosc 23 cells were cotransfected with pMT107, a plasmid expressing His_6-tagged ubiquitin, plus either pCGN control plasmid (lanes *1,3*) or pCGN-Abi 2 plasmid encoding HA-tagged human Abi 2 (lanes *2,4*). Total cell lysates (lanes *1,2*) or ubiquitin substrate conjugates that were affinity precipitated by Ni^{2+} chelate chromatography (lanes *3,4*) were subjected to Western blot analysis with anti-HA monoclonal antibody. The position of ubiquitinated Abi 2 is indicated by an arrowhead.

presence of an expression plasmid encoding His_6-tagged ubiquitin (Treier et al. 1994). The His_6-tagged ubiquitinated proteins were purified by Ni–agarose chromatography (Treier et al. 1994; Aberle et al. 1997) and subjected to Western blot analysis with monoclonal antibody to HA to detect the HA-tagged Abi 2 protein. As shown in Figure 3B, Abi 2 is ubiquitinated.

Oncogenic Src tyrosine kinase down-regulates Abi expression

We then wanted to test whether Abi proteins could also be down-regulated by expression of other oncogenic tyrosine kinases such as v-Src. A BaF3 cell line transfected with a zinc-inducible *v-Src* expression plasmid (Canman et al. 1995) was used in this experiment. The cells were treated with or without zinc for 8 hr to induce v-Src expression and cell lysates were subjected to Western blot analysis with either Abi (Fig. 4A, top) or v-Src (Fig. 4A, bottom) specific antibodies. Expression of v-Src was increased dramatically with the addition of zinc and the increased expression of v-Src correlated with a dramatic reduction of Abi expression (Fig. 4A, cf. lane 3 to lane 4). Thus, like Bcr–Abl, the oncogenic v-Src tyrosine kinase also down-regulates Abi expression in BaF3 cells. To determine whether the v-Src induced down-regulation of Abi expression is mediated by the ubiquitin–proteasome degradation pathway, we examined the effect of LLnL on Abi expression in v-Src-expressing BaF3 cells. v-Src-transfected cells were incubated with zinc in the presence or absence of LLnL. Zinc induced expression of v-Src (Fig. 4B, lanes 5,6), regardless of the presence or absence of LLnL. Down-regulation of Abi expression in cells treated with LLnL was completely inhibited compared with cells without LLnL treatment (Fig. 4B, cf. lanes 5 and 6). This result demonstrates that v-Src down-regulates Abi expression through ubiquitin-mediated proteolysis.

Bcr–Abl-mediated down-regulation of Abi expression is Ras independent

Oncogenic Bcr–Abl proteins elicit cellular transformation through multiple signal transduction pathways (Gotoh and Broxmeyer 1997). Previously, we and other investigators have shown that Ras function is activated

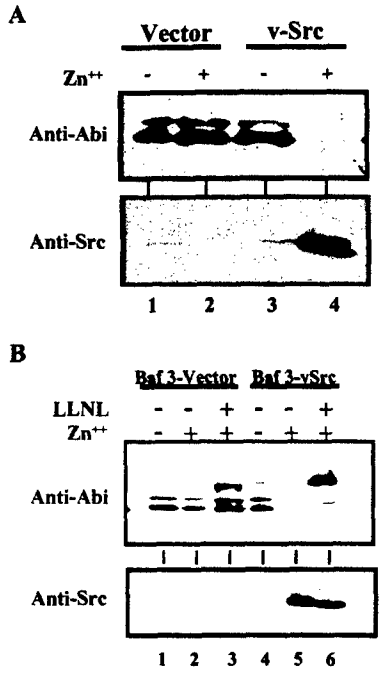

Figure 4. v-Src down-regulates Abi expression through an ubiquitin-dependent proteolysis pathway. (*A*) Oncogenic v-Src tyrosine kinase down-regulates Abi expression. BaF3 cells transfected by vector alone (lanes *1,2*), or zinc-inducible v-Src-expressing vector (lanes *3,4*) were treated with or without 75 μM $ZnCl_2$ for 8 hr, as indicated. Total cell lysates (2×10^6) were subjected to Western blot analysis with either 5421 anti-Abi antibodies (*top*) or anti-v-Src antibodies (*bottom*). (*B*) Proteasome inhibitor LLnL inhibits v-Src-induced down-regulation of Abi expression. BaF3 cells transfected by vector alone (lanes *1–3*) or zinc-inducible v-Src expression vector (lanes *4–6*) were treated with (lanes *3,6*) or without (lanes *1,2,4,5*) 50 μM LLnL for 1 hr. $ZnCl_2$ (75 μM) was then added as indicated, and cells were incubated for an additional 7 hr. Cells (2×10^6) were lysed in SDS sample buffer and subjected to Western blot analysis with either 5421 anti-Abi (*top*) or anti-v-Src antibodies (*bottom*).

in Bcr–Abl-transformed cells and it is a necessary component for Bcr–Abl-mediated transformation (Pendergast et al. 1993; Puil et al. 1994; Cortez et al. 1995, 1996). Studies on photoreceptor cell differentiation of the *Drosophila* eye have shown that activation of the Ras/Map kinase-signaling cascade results in the ubiquitin-mediated degradation of Tramtrack (TTK) (Li et al. 1997; Tang et al. 1997), a transcriptional repressor of neuronal cell fates, as well as of the transcription factor YAN (Rebay and Rubin 1995), a general inhibitor of differentiation of many cell types in the *Drosophila* eye. Therefore, we examined whether the Bcr–Abl-mediated down-regulation of Abi expression is Ras dependent. We used p185 and p210 Bcr–Abl-transformed 32D cells that coexpress a dominant-negative form of Ras, Ras Asn 17 (Feig and Cooper 1988), under the control of a glucocorticoid responsive promoter. Previously, we have shown that the inducible expression of dominant- negative Ras Asn 17 blocks Bcr–Abl from activating Ras in these cells (Cortez et al. 1996). Cells were treated with dexamethasone for 24 hr to obtain high-level expression of dominant-negative Ras Asn 17. The expression of Bcr–Abl (data not shown), dominant -negative Ras (Fig. 5A, lanes 1–4), and Abi proteins (Fig. 5A, lanes 5–11) was evaluated by Western blot analysis. Despite overexpression of dominant-negative Ras Asn 17, the expression of Abi proteins in the dexamethasone-treated cells (Fig. 5A, lanes 9,11) is down-regulated to low levels similar to nontreated cells (Fig. 5A, lanes 8,10) or control cells that express Bcr–Abl alone (Fig. 5A, lanes 6,7). Because Raf is an immediate downstream component of Ras in the Ras/Map kinase signaling cascade, we tested whether the enforced expression of an activated Raf protein kinase would elicit the down-regulation of Abi expression in BaF3 cells. A BaF3 cell line that inducibly expresses an activated form of human c-Raf (c-Raf–BXB) (Canman et al. 1995) from a zinc-responsive promoter was grown in the presence or absence of zinc. Zinc induced the expression of c-Raf-BXB (Fig. 5B). Consistent with the findings in Figure 5A, the enforced expression of the activated Raf did not affect the expression of Abi protein (Fig. 5B, cf. lane 3 to lane 4). Taken together, our results demonstrate that down-regulation of Abi expression by oncogenic Bcr–Abl is Ras and Raf independent.

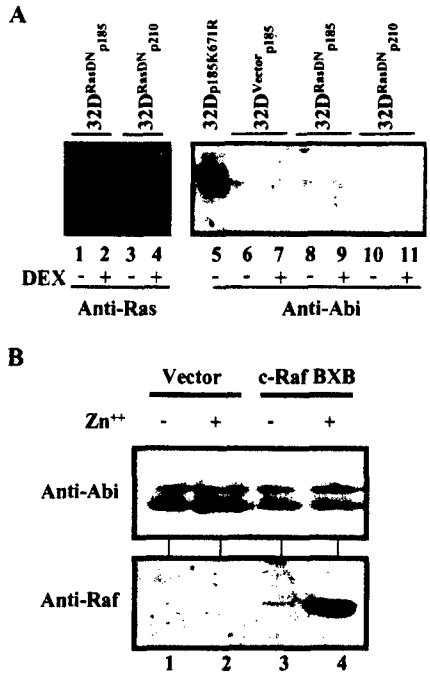

Figure 5. Bcr–Abl-mediated down-regulation of Abi expression is independent of Ras signaling. (*A*) Expression of a dominant-negative Ras, Ras Asn 17, failed to block Bcr–Abl-mediated down-regulation of Abi expression. 32D cells expressing a control plasmid ($32D^{Vector}$, lanes 6,7) or a plasmid inducibly expressing Asn 17 Ras ($32D^{RasDN}$, lanes *1–4* and *8–11*) were infected with retroviruses carrying either the *p185Bcr–Abl* (lanes *1,2,* and *6–9*) or *p210Bcr–Abl* (lanes *3,4,10,11*) oncogenes. The cells were treated with or without 50 nM dexamethasone for 24 hr, as indicated, to induce the expression of Ras Asn 17. A control 32D cell line infected with a retrovirus encoding a kinase-deficient *p185Bcr–Abl* mutant was also included (lane *5*). Cells (2×10^6) were lysed in SDS sample buffer and subjected to Western blot analysis with either anti-Ras antibody (pan Ras, Santa Cruz) (lanes *1–4*) or 5421 anti-Abi antibody (lanes *5–11*) as indicated. (*B*) Expression of an activated form of c-Raf does not down-regulate Abi expression. BaF3 cells transfected with either vector alone (lanes *1,2*), or an inducible expression vector encoding activated c-Raf, c-Raf–BXB, were treated with or without 75 μM $ZnCl_2$ for 8 hr as indicated. Cells (2×10^6) were lysed in SDS sample buffer and subjected to Western blot analysis using 5421 anti-Abi antibody (*top*) or anti-Raf antibody (Santa Cruz, *bottom*).

The expression of Abi proteins is lost in cell lines and bone marrow cells from Philadelphia chromosome-positive leukemia patients

The finding that Bcr–Abl down-regulates the expression of Abi proteins in BaF3 cells and 32D cells prompted us to test whether the Abi protein levels are also down-regulated as a consequence of Bcr–Abl expression in primary bone marrow cells, the natural target of the oncogenic Bcr–Abl tyrosine kinase. Mouse bone marrow cells were infected with Bcr–Abl and the expression of Abi proteins was examined by Western blotting (Fig. 6A, bottom). Consistent with the results observed in BaF3 and 32D cells (Fig. 6A, lanes 1,2), infection of bone marrow cells with Bcr–Abl retrovirus results in a loss of Abi expression (Fig. 6A, cf. lanes 3 and 4, bottom) that correlates with the expression of Bcr–Abl protein (Fig. 6A, top). Then we tested whether the expression of Abi proteins is also reduced in bone marrow cells from patients with Ph^1-positive human leukemias. The Abi proteins were shown to be expressed in bone marrow cells from either normal human samples or a Ph^1-negative leukemia patients (Fig. 6B, lanes 3,4). In contrast, Abi protein expression was lost in bone marrow cells from a Ph^1-positive patient with ALL or from a Ph^1-positive patient with CML in the blast crisis phase of the disease (Fig. 6B, lanes 1,2). K562 and MEG01 are cell lines derived from patients with CML in the blast crisis phase of the dis-

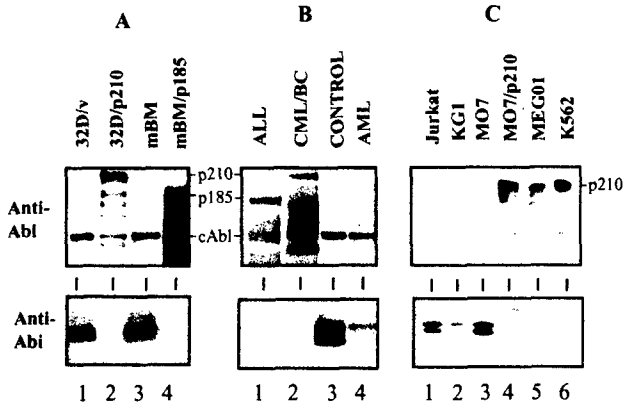

Figure 6. Expression of Abi proteins is lost in Bcr–Abl-transformed primary bone marrow cells and cells from Ph[1]-positive leukemia patients. Western blots were performed to compare Abl immunoreactivity (*top*) with Abi protein expression (*bottom*) in murine cells, human leukemia samples, and human cell lines. (*A*) 32D cells were infected with retroviral constructs containing vector alone (32D/v, lane *1*) or p210 Bcr–Abl (32D/p210, lane *2*). Primary mouse bone marrow (mBM, lane *3*) was infected with a retroviral construct containing p185 Bcr–Abl (mBM/p185, lane *4*) and analyzed after 18 days of selection with G418. (*B*) Bone marrow was obtained from patients with Ph[1]-positive acute lymphoblastic leukemia (ALL, lane *1*), or with Ph[1]-positive chronic myelogenous leukemia in blast crisis (CML/BC, lane *2*), and was compared to normal bone marrow (control, lane *3*) and bone marrow obtained from a patient with Ph[1]-negative acute myelogenous leukemia (AML, lane *4*). (*C*) Ph[1]-negative human leukemia cell lines (Jurkat and KG1, lanes *1,2*) and human myeloid cell line MO7 (lane *3*) were compared to Ph[1]-positive cell lines (MEG01 and K562, lanes *5,6*) or to a Ph[1]-negative cell line infected with retrovirus containing p210 Bcr/Abl (MO7/p210, lane *4*).

ease. Therefore, we compared these cell lines with a human myeloid cell line (MO7e), a cell line from a Ph[1]-negative acute myelogenous leukemia patient (KG1), and a cell line from a T-cell leukemia patient (Jurkat), for the expression of Abi proteins. Examination of the mRNAs for *abi 1* and *abi 2* by reverse-transcriptase PCR revealed that they are present in all of these cell lines (data not shown). As shown in Figure 6C, expression of Abi proteins was undetectable in those cells from the Ph[1]-positive leukemia patients, whereas Abi proteins are present in the other cell lines (Fig. 6C, lanes 5,6). Consistently, the down-regulation of Abi expresion correlates with the expression of oncogenic Bcr–Abl (Fig. 6C, upper). Taken together, these data suggest that loss of Abi protein expression may be a component in the progression of Bcr–Abl-positive leukemias.

Discussion

We have identified a novel pathway downstream of the oncogenic Abl and Src nonreceptor tyrosine kinases that targets the destruction of the Abl-interacting Abi family of proteins through the ubiquitin–proteasome pathway. The down-regulation of the Abi proteins requires the tyrosine kinase activity of Abl, and it is independent of the Ras–Raf pathway. Significantly, the degradation of the Abi proteins appears to be selective. Other molecules known to be degraded by the ubiquitin-dependent proteolysis pathway in response to extracellular signals or cell cycle progression, such as IκBα and the cyclin-dependent kinase inhibitor p27, are not affected by expression of oncogenic forms of Abl (Reuther et al. 1998; Z. Dai and A.M. Pendergast, unpubl.). It is likely, however, that additional proteins may be targeted for ubiquitin-dependent degradation after expression of the Abl and Src oncogenic tyrosine kinases.

It has become increasingly apparent that ubiquitin-dependent proteolysis of specific proteins is a highly regulated process. Linkage of ubiquitin to proteins that display a distinct degradation signal, results in the destruction of the ubiquitin–protein conjugate by the 26S proteasome (Varshavsky 1997). The conjugation of ubiquitin to the target protein involves a series of steps that begin with the formation of a thioester bond between ubiquitin and the ubiquitin-activating enzyme (E1). Ubiquitin is then transesterified to an ubiquitin-conjugating enzyme (UBC or E2) and subsequently transferred to the target protein, usually with the involvement of an ubiquitin protein ligase (E3). The latter is the component of the ubiquitin conjugation system that is involved in substrate recognition (Varshavsky 1997). Several ubiquitin-dependent degradation signals have been identified to date. Regulated destruction of target proteins is usually dependent on phosphorylation, interaction with specific proteins, or both. Most of the phosphorylation-regulated degradation signals identified to date are mediated by serine/threonine kinases (Rebay and Rubin 1995; Henchoz et al. 1997; Maniatis 1997; Verma et al. 1997). Although the tyrosine kinase activity of Bcr-Abl is absolutely required for Abi degradation, it is not clear at present whether direct tyrosine phosphorylation of the Abi proteins is critical for their proteolytic degradation. It is possible that the activated tyrosine kinases may induce Abi degradation through the phosphorylation of serine/threonine residues on Abi by protein kinases activated downstream of the oncogenic tyrosine kinases. Alternatively, the activated tyrosine kinases may induce the formation of a complex between Abi proteins and specific cellular proteins that target Abi for degradation. These two types of degradation signals are not mutually exclusive. Multiple serine, threonine, and tyrosine residues are found in the Abi proteins that may be phosphorylated by various protein kinases. Abi protein also contain sequences rich in proline, glutamic acid, serine, and threonine, designated as PEST, which have been found in many proteins that are targeted for ubiquitin-dependent degradation (Deshaies 1995; Rechsteiner and Rogers 1996). However, PEST sequences alone are not sufficient to identify those proteins that are targets of ubiquitin-dependent degradation (Varshavsky 1997). Extensive mutagenesis of the Abi proteins is necessary to identify those residues critical for their ubiquitin-dependent degradation. The availability of an in vitro degradation assay for the Abi proteins (Fig. 2) will facilitate the identification of the residues on Abi important for ubiquitin-de-

pendent degradation and will permit the isolation of the protein recognition complex that targets Abi for degradation by the proteasome.

The finding that Abi protein expression is lost in cells after expression of the transforming Bcr–Abl and v-Src tyrosine kinases, together with the discovery that Abi proteins are absent in cell lines and bone marrow cells isolated from patients with aggressive Bcr–Abl-positive leukemias, suggests that loss of Abi proteins by the ubiquitin–proteasome pathway may be a component in the progression of Bcr–Abl-positive leukemias and possibly other cancers. The irreversible nature of proteolysis makes this process uniquely suited for the elimination of growth inhibitory molecules during tumor progression. Indeed, several tumor-suppressor and growth-inhibitory proteins have been shown to be degraded by ubiquitin-dependent proteolysis. Among these is the p53 tumor-suppressor protein that is targeted for degradation by the human papilloma virus E6 oncoprotein (Scheffner et al. 1990) and the cellular Mdm 2 protein (Haupt et al. 1997; Kubbutat et al. 1997). Similarly, SHP-1, a protein tyrosine phosphatase that is implicated in receptor-mediated inhibitory signals, is targeted for ubiquitin-dependent degradation by an activated form of the Kit receptor tyrosine kinase (Piao et al. 1996). More recently, another link between tumor progression and increased proteasome-dependent degradation was provided by the finding that the cell cycle inhibitor p27 is targeted for ubiquitin-dependent degradation in aggressive colorectal carcinomas (Loda et al. 1997). These examples show that selective degradation of proteins that participate in the control of growth inhibitory pathways represents an alternative mechanism for their inactivation without the involvement of mutations or deletions in the corresponding genes.

Although our findings and those of other published reports are consistent with the hypothesis that Abi proteins function as growth inhibitors/tumor suppressors, an alternative role for these proteins, which cannot be ruled out at the present time, is that Abi proteins are downstream substrates of the oncogenic tyrosine kinases. The phosphorylated Abi proteins may transduce a signal from the oncogenic tyrosine kinases. Abi activation may be coupled to Abi destruction through the ubiquitin–proteasome pathway. The tightly coupled activation and the proteasome-dependent destruction of a protein has been documented for the p58 component of the yeast kinetochore Cbf 3 protein complex (Kaplan et al. 1997). The p58 protein is activated by phosphorylation as assayed by DNA-binding activity and subsequently it is degraded by the proteasome in a ubiquitin-dependent step. The phosphorylation and degradation of p58 are tightly coupled events that require the product of the *SKP1* gene p23 Skp1 (Kaplan et al. 1997). It has been proposed that p23 Skp1 functions as an adaptor that recruits a protein kinase by binding to both p58 and the unknown kinase. Also, p23 Skp1 is a component of the E3 ubiquitin ligase complex that targets p58 to the proteasome machinery. In this manner p58 is regulated positively by phosphorylation and regulated negatively by ubiquitin-dependent proteolysis. Linked activation and negative regulation of cellular proteins in response to phosphorylation has also been reported in mammalian cells for the STAT 1 transcription factor (Kim and Maniatis 1996). Activated STAT 1 has been shown to be regulated negatively by the ubiquitin–proteasome pathway. Tyrosine phosphorylation of STAT 1, which is induced by treatment of cells with interferon-γ, is required for its nuclear translocation and activation of transcription and it is also required for STAT 1 ubiquitination and subsequent degradation. A similar role for the phosphorylation of Abi proteins may exist in cells transformed by the activated Abl an Src tyrosine kinases.

The finding that Abi proteins are targeted for degradation by both oncogenic Abl and Src suggests that the activities of these two tyrosine kinases may be linked or alternatively, that they may regulate independently the function of specific common target proteins such as Abi. The data also raise the possibility that Abi proteins may be targeted for degradation in other human cancers where the Src family of nonreceptor tyrosine kinases are activated constitutively. Future work will be geared toward elucidating the cellular components of this novel pathway and the identification of additional targets of ubiquitin-dependent degradation triggered by the activity of oncogenic protein tyrosine kinases.

Materials and methods

Cell culture and retroviral infection

BaF3 cells and 32D cells were grown in RPMI containing 10% fetal calf serum (FCS) and 10% WEHI-conditioned media (WEHI-CM) as a source of IL-3. Stable mass populations of cells expressing *bcr–abl* transgenes were generated by retroviral infection as previously described (Cortez et al. 1995). BaF3 cell lines expressing v-Src and c-Raf-BXB (Canman et al. 1995) were grown in RPMI containing 10% FCS and 10% WEHI-CM. To induce the expression of v-Src and c-Raf-BXB, cells were treated with 75 μM $ZnCl_2$ for 8 hr. To inhibit ubiquitin-dependent proteolysis, cells were treated with either 10 μM lactacystin or 50 μM LLnL for 8 hr. Cells were washed once with PBS and lysed directly in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol] for Western blot analysis.

Anti-Abi antibodies

Rabbit polyclonal antibodies 5421 and 4575 anti-Abi were raised to a recombinant GST–Abi 2Δ1-100 fusion protein and a synthetic Abi 2 peptide (amino acids 318–329), respectively. Antibodies were affinity purified by standard techniques (Harlow and Lane 1988).

RNA analysis

abi 1 and *abi 2* mRNA levels were determined by RNase protection assays. Total RNA was isolated from BaF3 cells using TRIzol reagent (GIBCO BRL) or the RNeasy Mini Kit (Qiagen) as directed by each of the manufacturers. The protocols for these two methods were followed as directed. Antisense probes for *abi 1* and *abi 2* mRNA were generated by in vitro transcription with T3 RNA polymerase (Stratagene). *abi 1* probe was transcribed from a linearized template containing the T3 promoter

from the pPCR-Script Amp SK(+) plasmid (Stratagene) and a 244-nucleotide fragment of *abi 1* cDNA. *abi 2* probe was generated from linearized pBlueScript II SK +/- (Stratagene) plasmid containing a 435-bp fragment of *abi 2* mouse genomic DNA composed of a 48-bp intronic region and 397 nucleotides from a single exon. The probe for mouse GAPDH RNA was generated from the pTRI-GAPDH mouse linear fragment (Ambion) using T3 RNA polymerase. RNase protection experiments were carried out in accordance with instructions provided by the manufacturer (Ambion). Hybridization of probes to RNA protected the probes from digestion with RNase A plus T1 (Ambion). To detect *abi 2* by RNase protection, 30 µg of total RNA was used per lane. Twenty micrograms of RNA was used for *abi 1* and 10 µg of RNA was used for *GAPDH*. After digestion, protected probes for *abi 1*, *abi 2*, and *GAPDH* were analyzed in separate lanes of 6% acrylamide/urea gels. ^{35}S-Labeled protected fragments were detected by autoradiography.

Analysis of Abi expression in bone marrow cells

P185-expressing primary mouse bone marrow cells were generated by retroviral infection as described (Cortez et al. 1995). Mouse bone marrow was obtained by flushing the femurs of 3- to 4-week-old male BALB/c mice (Charles River Labs) with IMDM containing 2% FCS and either was used for retroviral infection followed by G418 selection as described (McLaughlin et al. 1989), or processed through two rounds of ammonium chloride lysis, lysed in 2× SDS sample buffer and used directly for Western blot analysis using antibodies against the Abl kinase domain (8E9, Pharmingen) or Abi-2. Bone marrow samples from leukemia patients were obtained from the SWOG Human Tissue Bank (University of New Mexico) or the Duke Human Tissue Bank (Duke University Medical Center). Normal bone marrow was obtained after informed consent from patients undergoing autologous bone marrow transplantation for nonhematological malignancies. After Ficoll density gradient centrifugation (Sigma), light density human bone marrow cells were resuspended in PBS containing 0.01 mg/ml aprotinin (Boehringer Mannheim), 5 mM benzamidine (Sigma), and 1 mg/ml AEBSF ("Pefabloc SC" Boehringer Mannheim), then lysed with 2× SDS sample buffer for Western blot analysis. Mouse myeloid and mouse and human bone marrow samples were normalized to give roughly equal levels of Abl immunoreactivity.

In vitro degradation assay

^{35}S-Labeled human Abi 2 was produced using RRL and plasmid pT7T3-Abi 2 with a coupled transcription–translation kit (Promega). The transcription–translation reaction was performed at 30°C for 120 min in the presence of [^{35}S]methionine, as indicated by the manufacturer. To produce unlabeled p185Bcr–Abl, the transcription–translation reaction was performed using plasmid pGEMp185Bcr–Abl in the presence of a complete amino acid mix. Protein stability was analyzed by incubating 3 µl of ^{35}S-labeled Abi 2 in 50 µl of degradation mix [33% RRL, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl, 2 mM dithiothreitol, 1 mM ATP, and 2 mM methionine] at 37°C in the presence or absence of 3 µl of p185Bcr–Abl-containing lysate. Where indicated, 1 mM ATP was substituted with 2 mM ATPγS. The reaction was stopped by addition of an equal volume of 2× SDS sample buffer and analyzed by SDS-PAGE.

Affinity precipitation

Purification of His-tagged ubiquitin expressed transiently in Bosc 23 cells was performed following the method of Treier et al. (1994). Briefly, 48 hr after transfection cells were lysed with 1 ml of GTN buffer per 60-mm dish [6 M guanidinium–HCl, 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM imidazole, 0.1% TX-100]. The lysate was sonicated with a microtipped sonifier at setting 4 for 20 sec to reduce viscosity. Fifty microliters of Ni^{2+}–NTA–agarose beads (Qiagen) was added and mixed for 4 hr at room temperature. The beads were successively washed with the following solutions (pH 8.0): 1 ml of GTN; 1 ml of 8 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 8 M urea, 20 mM Tris-HCl, 1 M NaCl, 0.1% TX-100; 1 ml of 4 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; 1 ml of 1 M urea, 20 mM Tris-HCl, 200 mM NaCl, 0.1% TX-100; and 1 ml of 20 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole, 0.1% TX-100. The bound His_6-tagged ubiquitin substrate complexes were analyzed by SDS-PAGE and Western blotting.

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DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754